THE RESPONSE OF SOME CROP PLANTS TO PHYTOHORMONES AND SELECTED ELEMENTS

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This dissertation is humbly dedicated to the memory of my father

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CERTIFICATE

This is to certify that **MS.SHAZIA ALVI** has worked under my supervision for the M.Phil. degree in Botany.She has fulfilled all conditions required to supplicate the M.Phil. degree. I therefore, approve that she may submit her dissertation entitled " ***The response of some crop plants to phytohormones and selected elements***".

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Supervisor of Research

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(SHAZIA ALVI)

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Introduction

CHAPTER-1

INTRODUCTION

The seed plays a unique role in the life history of the plant. The success with which the new individual is established is largely determined by the physiological and biochemical features of the seed. The seed which develops from the fertilized ovule is usually comprised of (1) the testa - the product of one or both integuments of the ovule; (2) the perisperm-derived from the nucellus; (3) the endosperm, produced as a result of the fusion between one male generative nucleus and the two polar nuclei to form the triploid endosperm nucleus and (4) the embryo, the result of fertilization of the oospore by a male nucleus.

Seeds characteristically contain large amount of food reserve which supports the growth and development of the seedling until it can establish itself as a photosynthesizing autotrophic plant. These reserves are laid down as discret, intracellular bodies and include lipid, protein, carbohydrate, organic phosphate and various inorganic compounds. Some nutritionally undesirable reserves such as alkaloids, lectins, proteinase inhibitors, phytin and raffinose oligosaccharides are also present.

Germination is a series of events which take place when dry quiescent seeds imbibe water resulting in an increase in metabolic activity and the initiation of seedling from the embryo. The five stages involved in the seed germination and seedling growth are (1) imbibition and

hydration of protoplasm, (2) leakage of solutes, (3) activation and synthesis of enzymes (4) metabolism of storage products, their subsequent transport and synthesis of new materials and (5) cell elongation leading to the emergence of radicle.

Before germination process starts the seed takes up a large quantity of water. Many seeds placed under optimum favourable conditions for germination show a triphasic pattern of water uptake. Phase I occurs equally well in dead and living tissues and is, therefore, independent of the metabolic activity of the seed. Phase II is the period of active metabolism, in preparation for germination in non-dormant seeds of active metabolism in dormant seeds. Phase III is associated with germination and subsequent growth. During the initial stages of imbibition several substances like sugars, organic acids, phenols, inorganic phosphates and potassium leach out of the seed into the soil which might put the embryo under stress when the solubilization of stored food has just started. On hydration, during germination, the seed actively respires which supplies the energy (ATP); reducing power (NADH and NADPH) and carbon skeleton. Initially, there is a sharp increase in O_2 consumption, which can be partly attributed to the activation and hydration of mitochondrial enzymes involved in the krebs cycle and electron transport chain. The hydration of the seed is completed when oxygen uptake is stabilized and all pre-existing enzymes are activated. Shortly, following the absorption of water by the seed, enzyme activity begins to appear. Numerous enzymes such as lipases, proteinases, phosphatases, hydrolases, calmodulin, carboxypeptidases, and others (Bewly and Black 1985; Mayer and

Poljakoff-Mayber 1989; Coccuni and Negrini 1991; Washio and Ishikawa 1992; Bernier and Ballance 1993; Bernhardt *et al.* 1993) are either activated or synthesized a fresh. After the breakdown of the storage compounds into simpler chemical substances, they are transported from the endosperm cotyledons to the embryonic axis. These compounds are utilized in the production of new enzymes, structural materials, regulatory compounds, plant growth substances, and nucleic acids which carry out and regulate cell junctions and are consumed in the synthesis of new material. (Davies and Slack 1981; Mayer and Poljakoff-Mayber 1989).

The involvement of various plant hormones alone and in combination is based on the correlation of their endogenous concentration and the germination state. Gibberellins are a class of phytohormones which are most directly implicated in the germination process of the seeds. However, the involvement of the other major phytohormones (IAA, cytokinin, ABA, ethylene) cannot be over sighted. The role of auxins is not specifically assigned during the course of seed germination but it is actively involved (Bewley and Black, 1985).

The seeds are loaded with a sufficient quantity of cations and anions where calcium and potassium are not only involved as structural units of the membranes but also regulate its functioning. They also take active part in regulating cellular metabolism by controlling the activation and *de novo* synthesis of certain proteins (Bewley and Black, 1985). There is always a cumulative response of the cellular metabolism to the calcium and other factors (light and phytohormones). Therefore, the concentration of Ca^{2+},

at the level of the cytosol, may be altered for the signalling purposes by transiently opening the calcium channels in the plasma membrane or an intracellular membrane through the use of phytohormones.

The present study was aimed to elucidate the interactive effect of exogenously applied phytohormone (IAA) and the ions (Ca^{2+} and K^{+}) in regulating the seed metabolism at various intervals of seed germination to correlate their role.

Chapter-2

Review of Literature

REVIEW OF LITERATURE

CHAPTER-2

REVIEW OF LITERATURE

2.1 Introduction

The hydration of the mature seed induces the synthesis/release of hydrolytic enzymes to initiate the solubilization of reserve food material. The simpler substances released are either respired to gain energy or absorbed in the synthesis of cellular constituents, at the level of the embryo, facilitating its development and growth. However, the whole process of germination is an accumulative expression of various factors, borned either within the seed or outside it. The level of hormonal reserves in the seed is one of the major regulators of the whole process of germination.

In a number of isolated cases, hormones are found to promote germination of the seeds. The exogenous application of GA improved the pre cent germination of the seeds of *Eleusine coracana*, Sesame,*Brassica juncea* (Mohanty and Sahoo, 1992), *Vigna mungo* (Baghel and Yadava, 1991; Sharma and Saran, 1992), *Vigna radiata* (Patel and Saxena, 1994). The involvement of the GA is further strengthen by the observation of Thomas (1989), who could overcome the inhibitory effect of GA biosynthesis inhibitors by subsequent addition of solution of GA_4 and GA_7 or the GA-active compound (1-3 chlorophthalimide). It was therefore, suggested that the biosynthesis of gibberellin is essential for breaking the dormancy in celery seeds.

The cytokinin is present in sufficient quantity in the developing seeds but its values decrease as the maturity reaches (Krishnamoorthy, 1993) and again the various forms increase as the germination advances (Saha and Sircar, 1996). Therefore, the seed treatment of green gram and black gram with kinetin enhanced germination (Patel and Saxena, 1994).

The mechanism involved in the action of ethylene, in seed germination, is not quite clear, however, in many species, exogenous ethylene or ethephon stimulates seed germination. Even ACC (1-aminocyclopropane-1-carboxylic acid) could improve seed germination but less efficiently (Kepczynski and Kepczynska, 1997). Similarly, auxins (IAA, NAA and IBA) are also recognized to effect the germination of the seeds of *Dolichos biflorus* (Nanda and Nanda, 1995), *V. radiata* and *V. mungo* (Baghel and Yadava, 1991; Patel and Saxena, 1994) in *Vigna mungo* and *Arachis hypogoea* (Manikanand and Hakim, 1999).

The contents of macronutrients differ qualitatively as well as quantitatively between different seeds (Bewly and Black, 1985), therefore, soaking the seeds of *V. mungo* in the extract of cow dung or 50 ppm solution of phosphorus for 12 hours improved germination percentage, compared with those soaked in water (Joseph, 1991). However, under saline (NaCl, KCl, KNO_3, $CaCl_2$ or $CaNO_3$) conditions the process of germination was either delayed or slowed down in the seeds of *Phaseolus mungo* (Sharma and Saran, 1994), wheat (Kamboh, *et al.*, 2000). Contrary to this, the hypochlorides of sodium (NaClO) and calcium ($CaClO_2$) favoured the germination of the orchid seeds (Miyoshi and Mii, 1998).

The response of the seeds to various trace elements varied. Vijaya and Punnuswamy (1997) treated the seeds of *Vigna mungo* and cowpea with 100 mg/kg of $ZnSO_4$, $MnSO_4$ or Na_2Mo_4 singly or in various combinations. The treatment augmented the germination of the seeds of *V. mungo* but had no effect on cowpea. Similarly, the other cultivors gave a positive response to Mn but Hg (Nandi *et.al.*, 1995) and fluoride (Dhananjayanath *et.al.*, 1993) decreased/inhibited the germination.

2.2 Metabolic changes associated with germination

Germination is the process which culminates in the emergence of the radicle. Therefore, one has to concentrate on the phenomenon going on in the first few hours where the cell enlargement is the major event and is influenced by the auxins and other chemicals favouring overall germination of the seed. It has to be necessarily associated with the synthesis of the proteins, as wall components and critical enzymes required in the regulation of both metabolic and catabolic processes.

Nitrate reductase (NR) is a phytochrome mediated enzyme and its activity is facilitated by light. Out of the four forms of NR in *Sinapis alba* the appearance of NR_1, NR_2 and NR_3 was strongly stimulated by red light whereas, NR_4 required the presence of continuous light (Schuster *et.al.*, 1989). Light also effects the rate of nitrate absorption by increasing the activity of NRA. It was confirmed by inhibiting the activity of NR by tungstate, that eventually decreased nitrate absorption (Rao and Rains, 1976). The increased activity of NR, in the presence of light/nitrate, and glutamine synthase (GS) is a coordinated appearance attributed to the

de novo synthesis of the enzymes proteins but GS level being slightly above that of NR. It appears to be the safety measures that the accumulation of ammonia may be prevented (Weber *et.al.*, 1990). Apart from light, substrate (nitrate) does have promotive effect on NR activity (Schuster *et.al.*, 1989). In addition to phytochrome and substrate, phytohormones profoundly effect the activity of NR. While nitrate maintained a steady level, irrespective of the treatment. The level of NRA was found to be much higher in the aerial leafy part than in the rooting zone of the pea cuttings, fed with an aqueous solution of IAA or IBA, during early rooting stage (Ahmad, 1988). Moreover, both the uptake and activity of NR did not depend on the endogenous auxins of pea cuttings in the first few days of rooting (Ahmad, 1994). The other hormones (GA_3 and kinetin) induced a significant increase in the activity of NR and NiR (Premabatidevi, 1998).

α-amyase is a Ca-containing protein, synthesised on the rough endoplasmic reticulum of the cell. GA_3 and ABA among the hormones, are known to regulate α-amylase production in aleurone layer cells of *Hordeum vulgare* seeds. GA_3 increased and abscisic acid (ABA) decreased Ca-flux into the endoplasmic reticulum and the amount of Ca that accumulated in the ER of barley aleurone cells (Bush *et.al.*, 1993). Fluctuations in the endogenous level of GA_1 and ABA in the germinating seeds of *Hordeum vulgare* confirmed the hypothesis that GA and ABA regulate the induction of α-amylase. The level of GA_1 was highest on the second day while that of ABA was lowest on the first day of the seed germination (Kobayoshi *et.al.*, 1995). The embryo contributes in the

Hormones cause influx of Ca^{2+} into the cells through specific Ca^{2+} channels in the plasmamembrane raising the cytosolic level of the ion, triggering the cellular response (Lehninger *et.al.*, 1993; Ching and Choi, 1998). Calcium is implicated in ABA induced thermotolerance in *Zea mays* (Ming *et.al.*, 1998), improved cold tolerance in low temperature induced injuries in *Oryza sativa* (Ying *et.al.*, 1996). Similarly it mediates the activation of NAD^+ kinase by involving IAA (Lixia *et.al.*, 1999). It has also been suggested by Yael and Weiss (1999) that there is a relationship between intercellular level of Ca^{2+}, calmodulin, protein phosphorylation and dephosphorylation with GA iduced gene expression including chalcone synthase in *Petunia corolla*. The biological responses to the interaction effect of the calcium and the hormone may be through an increase in the level of calmodulin (CaM) protein which is quite evident in case of aleurone layer cells incubated in GA_3 + $CaCl_2$ but was reduced by ABA (Schurink *et.al.*, 1996). Moreover, indole acetic acid also stimulated protein secretion by activating Ca^{++} channels of plasmalemma vesicles (Mendvedev *et.al.*, 1999).

Chapter-3

Materials & Methods

MATERIALS AND METHODS

CHAPTER-3

MATERIALS AND METHODS

3.1 Proposed study

To achieve the objectives framed in chapter one, following studies were conducted to explore the response of seeds (*Cicer arietinum* cv. BG-256) to Indole-3-acetic acid (IAA), Ca^{2+} or K^{+} during the early stages of imbibition, under controlled conditions.

3.2 Seeds

The seeds of *Cicer arietinum* L. cv. BG-256 were procured from the National Seed Corporation Ltd. I.A.R.I. New Delhi. The healthy seeds were surface sterlized with 0.01% mercuric chloride solution followed by repeated washing with double distilled water (DDW).

3.3 Experiment

An experiment was conducted to study the interactive effect of IAA with Ca^{2+} or K^{+}.

(A) The surface sterilized seeds were soaked in distilled water and aqueous solutions of IAA (10^{-8}M), calcium chloride (4 or 8 μm of Ca^{2+}), potassium chloride (6 or 12 μM of K^{+}) to be used as control.

(B) The surface sterlized seeds were first allowed to imbibe in 10^{-8}M solution of IAA in sterilized petri plates for 6 hours and subsequently transferred, after being soaked with blotting paper, to different petri plates containing solutions of 4 and 8 μM of Ca^{2+} or 6 and 12 μM of K^{+} for

another six hours. These treated seeds were thoroughly washed with DDW to remove the adhering solutions and then transferred to sterlized petriplates containing cotton, moistened in DDW.

(C) Surface sterilized seeds were first allowed to imbibe in 4 and 8 μM of Ca^{2+} or 6 and 12 μM of K^{+} solutions for 6 hours and then transferred to 10^{-8} M solution of IAA for another 6 hours. These treated seeds were washed with DDW and transferred to sterlized petriplates containing cotton, moistened in DDW.

Seeds were allowed to germinate in BOD incubator at 28±2°C and sampled at 12, 24 and 36 hours of the soaking (which also include the duration of treatment).

The following characteristics were studied in the seed.

1. Nitrate reductase activity.

2. Nitrate content

3. Calcium and Potassium contents.

4. Total protein content.

3.4 Chemical analysis

3.4.1 Estimation of nitrate reductase activity

Nitrate reductase activity (NRA) in the seed was estimated according to the method of Jaworski (1971). Biological sample was cut into small pieces of which 200 mg was weighed and transferred into polythene vial (25 ml). 2.5 ml of phosphate buffer (appendix 1.1) 0.5 ml

of 0.2M potassium nitrate (appendix 1.2) solution and 2.5 ml of 5% isopropanol were added. At least two drops of chloromphenicol were also added to check bacterial growth in the test sample. The vial was incubated in the BOD incubator at 30±2°C for 2 hours, in the dark.

3.4.1.1 Colour development

In a test tube 0.4 ml of test extract and 0.3 ml each of sulphanilamide (appendix 1.3) and naphthyl ethylene diamine hydrochloride (NED-HCl, appendix 1.4) were added. A pink colour appeared. The sample was left for 20-30 minutes for maximum colour development. It was diluted upto 5 ml with DDW and each sample was read at 540 nm using "Spectronic-20" colorimeter. Calibration, for 100% transmittance, was done by using a blank consisting of 4.4 ml DDW and 0.3 ml each of sulphanilamide and NED-HCl.

Standard curve was plotted using graded concentrations of potassium nitrite. Optical density of the test extract was read on the calibrated curve and NRA was calculated in terms of nmoles NO_2 $g^{-1}h^{-1}$ FW.

3.4.2 Estimation of nitrate

The nitrate content was estimated following the method of Johnson and Ulrich (1950).

3.4.2.1 Preparation of powder

The samples were dried, over night, in an oven at 80°C. The grinding was done in an electric grinder and powder passed through a 72

mesh screen and stored in polythene vials for the estimation of nitrate. Each sample, before analysis, was again dried over night in an oven at 80°C on a clean sheet of paper. In the morning the samples were placed in a dessicator for 15 minutes for cooling under dry conditions.

3.4.2.2 Extraction and colour development

50 mg of the above powder was weighed and transferred to a dried centrifuge tube (25 ml) with the addition of 400 mg of calcium sulphate and 12.5 ml of DDW. The sample was centrifuged for 10 minutes, at 6000 rpm. the supernatant was transferred to a 50 ml conical flask containing 1 ml of 0.5% calcium carbonate suspension. The excess solution was evaporated on water-bath leaving the final volume to 5 ml only. 0.5 ml of H_2O_2 (30%) was added to the above solution and the flask was closed with a lid. After 5 minutes the lid was removed and the solution in the flask was heated further to dryness on a water-bath in order to remove the peroxide. The flask was cooled and 1.25 ml of phenol-di-sulphonic acid (appendix 2.1) was rapidly added with continuous stirring. 35 ml of distilled water was also added to this solution. Lastly, 3 ml of 50% ammonium hydroxide solution was pipetted into it, yellow colour developed. The transmittance was noted, after 15 minutes at 400 nm using a "Spectronic-20" colorimeter. A standard curve was plotted using the graded concentrations of standard potassium nitrate solution.

3.4.3 Estimation of proteins

Total protein level in the sample was determined following the method of Lowry *et.al.* (1951).

3.4.3.1 Extraction

50 mg of the oven dried powder was transferred to a mortar. Samples was grind with the addition of 1 ml of trichloroacetic acid (5%). It was transferred to a centrifuge tube with repeated washings and the final volume was made up to 5 ml with trichloroacetic acid. The material was centrifuged at 4000 rpm for 15 minutes and the supernatant was discarded. 5 ml of 1N sodium hydrxide (NaOH) was added to the residue. It was left for 30 minutes in a waterbath at 60°C. After cooling for 15 minutes, the mixture was centrifuged at 4,000 rpm for 15 minutes and the supernatant was collected in 25 ml volumetric flask. Volume was made up to the mark with the help of 1N NaOH and used for the estimation of total protein.

3.4.3.2 Colour development

One ml of NaOH extract was transferred to a test tube and 5 ml of reagent D (appendix 3.1) was added to it. The solution was mixed well and allowed to stand at room temperature for 15 minutes. 0.5 ml of folin phenol reagent (appendix3.2) was added rapidly with immediate mixing. The blue colour developed. The per cent transmittance of this solution was read at 660 nm using 'spectronic-20' colorimeter. A blank was run with each sample. The total protein content was calculated by comparing the optical density of each sample with a calibration curve plotted by taking known graded dilutions of a egg albumin.

3.4.4 Estimation of calcium and potassium

The following methodology was followed for the estimation of calcium and potassium in the samples.

3.4.4.1 Digestion of powder

100 mg of dry powder of the sample was taken in a 50 ml kjeldhal flask. To this 2 ml of concentrated sulphuric acid was added and the mixture was heated on a digestion rack, for 2 hours. The contents turned black in this duration. After cooling for 15 minutes, 0.5 ml of chemically pure H_2O_2 (30%) was added drop by drop till a clear solution was obtained. The peroxide digested material was transferred to a 100 ml volumetric flask with at least three washings with distilled water and the volume made upto the mark.

3.4.4.1.1 Estimation of calcium

Flame photometer was used to read the calcium content in the digested samples with the help of calcium filter. Per cent quantity of the element was calculated by comparing the readings on a standard curve plotted by using known dilution of calcium carbonate.

3.4.4.1.2 Estimation of potassium

Potassium content in the digested aliquot was directly estimated flame photometrically following the procedure explained by Singh (1988) by using potassium filter. A blank was run side by side. The reading was compared with a calibration curve plotted by using known dilutions of a standard potassium chloride solution.

3.5 Statistical analysis

The experimental data was analysed statistically following the procedures explained by Gomez and Gomez (1984).critical difference (C.D.) at the 5 % level of probability was calculated.

Chapter-4

Experimental Results

RESULTS

CHAPTER-4

RESULTS

4.1 Nitrate reductase activity

There is a consistent increase in the activity of nitrate reductase (NR) upto 36 hours of the imbibition (Table 1). The level of the enzyme increased significantly by the treatment. A maximum increase was recorded in the seeds soaked for 6 hours in 10^{-8}M of IAA only. The values were 50%, 95% and 77% more than the control at 12, 24 and 36 hours of the germination. Treating the seeds with either of the ions (Ca^{2+}, K^{+}) alone or in association with the auxin did not prove as good as the auxin alone. However, soaking the seeds first in IAA followed in the ionic solution for 6 hours each, proved better than otherwise. Within the two ions tested, Ca^{2+} proved better than K^{+} in association with IAA. The best combination being IAA+Ca^{++} (8 μm) where the values increased by 42%, 60% and 46% at the three samplings (12, 24 and 36 hours).

4.2 Nitrate content

The content of nitrate decreased linearly as the germination progressed (Table 2). The seeds soaked in water (control) had maximum level of the ion at all the stages of smapling. In all the cases where the seeds were treated with auxin alone or in any combination with Ca^{2+} or K^{+}, the nitrate concentration was significantly less than the control. However, the IAA (10^{-8}) alone was most effective in reducing (metabolizing) the nitrate level in the seeds.

Table 1. **The interaction effect of IAA (10^{-8}M) with Ca^{2+} (4 and 8 μM) or K^{+} (6 and 12 μM) on the nitrate reductase activity (nmol $NO_3h^{-1}g^{-1}FW$) in the seeds of *Cicer arietinum* L. cv. BG-256, at 12, 24 and 36 hours of the sampling.**

Treatments	Sampling time (Hours)		
	12	24	36
Control	200.11	288.40	359.03
IAA 10^{-8}M	300.17	565.03	635.66
Ca4 μM	247.20	341.37	405.10
Ca8 μM	268.35	383.45	441.49
K6 μM	229.54	305.45	369.25
K12 μM	258.97	349.30	410.15
Ca4 + IAA	260.35	396.65	471.30
Ca8 + IAA	274.13	443.45	510.25
K6 + IAA	251.45	370.13	440.35
K12 + IAA	263.66	405.10	460.29
IAA + Ca4	279.35	419.30	479.36
IAA + Ca8	284.68	461.25	525.14
IAA + K6	263.45	386.10	451.39
IAA + K12	269.38	429.35	485.25
C.D. at 5%	20.26	18.65	31.25

Table 2. **The interaction effect of IAA (10^{-8}M) with Ca^{2+} (4 and 8 μM) or K^{+} (6 and 12 μM) on the nitrate content (ppm) in the seeds of *Cicer arietinum* L. cv. BG-256, at 12, 24 and 36 hours of the sampling.**

Treatments	Sampling time (Hours)		
	12	24	36
Control	2.49	2.20	1.66
IAA 10^{-8}M	2.11	1.72	1.09
Ca4 μM	2.40	2.14	1.56
Ca8 μM	2.31	2.05	1.43
K6 μM	2.45	1.98	1.36
K12 μM	2.42	2.08	1.46
Ca4 + IAA	2.29	1.96	1.28
Ca8 + IAA	2.25	1.89	1.19
K6 + IAA	2.30	1.92	1.23
K12 + IAA	2.24	1.83	1.16
IAA + Ca4	2.22	1.79	1.21
IAA + Ca8	2.20	1.88	1.13
IAA + K6	2.18	1.83	1.18
IAA + K12	2.24	1.93	1.28
C.D. at 5%	0.16	0.21	0.29

4.3 Protein content

Total protein content exhibited a progressive increase with the advancement of germination (Table 3). All the treatments had a significant impact on the level of the protein, as compared with the water soaked, control. Throughout the germination period, the maximum protein content was recorded in the seeds pre-soaked in IAA (10^{-8}M) alone for 12 hours or 6 hours in IAA subsequently followed for 6 hours in Ca^{2+} (4 or 8 μM). The respective increase, over the control, is 23%, 15.75% and 17%, respectively. It was closely followed by K^{+} (12 μM) used alone or in association with IAA.

4.4 Calcium content

The per cent calcium content increased as the germination advanced (Table 4). All the treatments significantly increased the level of the ion, over the control. There was a parallel relationship between the calcium content of the seed and its contents in the soaking medium, with or without the presence of the auxin. Pre-soaking in potassium was most effective if the seeds were already treated with auxin.

4.5 Potassium content

The seeds possessed more potassium as the germination progressed and exhibited a significant response to the treatment (Table 5). There was a proportionate relationship between the potassium content of the seed as that of its concentration in the bathing medium. The ionic content was maximum in the seeds pre-treated with IAA (10^{-8}M) followed by 12 μM of potassium for 6 hours each. The calcium alone or in association with auxin had a comparable effect on the level of the potassium.

Table 3. **The interaction effect of IAA (10^{-8}M) with Ca^{2+} (4 and 8 μM) or K^{+} (6 and 12 μM) on the protein content (%) in the seeds of *Cicer arietinum* L. cv. BG-256, at 12, 24 and 36 hours of the sampling.**

Treatments	Sampling time (Hours)		
	12	24	36
Control	18.53	20.24	21.43
IAA 10^{-8}M	22.79	25.69	27.65
Ca4 μM	21.58	24.48	26.23
Ca8 μM	21.19	24.36	26.20
K6 μM	20.68	23.80	25.10
K12 μM	22.44	25.60	29.30
Ca4 + IAA	21.45	23.85	24.93
Ca8 + IAA	21.68	24.15	25.90
K6 + IAA	21.25	23.90	25.35
K12 + IAA	22.13	25.03	27.02
IAA + Ca4	23.00	25.85	27.80
IAA + Ca8	23.09	26.25	29.89
IAA + K6	21.68	24.68	26.45
IAA + K12	22.68	25.32	27.25
C.D. at 5%	0.98	0.79	1.35

Table 4. **The interaction effect of IAA (10^{-8}M) with Ca^{2+} (4 and 8 μM) or K^{+} (6 and 12 μM) on the calcium content (%) in the seeds of *Cicer arietinum* L. cv. BG-256, at 12, 24 and 36 hours of the sampling.**

Treatments	Sampling time (Hours)		
	12	24	36
Control	0.125	0.132	0.137
IAA 10^{-8}M	0.139	0.143	0.147
Ca4 μM	0.156	0.159	0.163
Ca8 μM	0.169	0.174	0.179
K6 μM	0.143	0.149	0.154
K12 μM	0.145	0.151	0.156
Ca4 + IAA	0.168	0.174	0.178
Ca8 + IAA	0.170	0.188	0.191
K6 + IAA	0.148	0.151	0.156
K12 + IAA	0.147	0.153	0.162
IAA + Ca4	0.158	0.170	0.178
IAA + Ca8	0.180	0.181	0.196
IAA + K6	0.153	0.159	0.163
IAA + K12	0.152	0.157	0.166
C.D. at 5%	0.011	0.013	0.009

Table 5. **The interaction effect of IAA (10^{-8}M) with Ca^{2+} (4 and 8 μM) or K^{+} (6 and 12 μM) on the potassium content (%) in the seeds of *Cicer arietinum* L. cv. BG-256, at 12, 24 and 36 hours of the sampling.**

Treatments	Sampling time (Hours)		
	12	24	36
Control	0.532	0.546	0.553
IAA 10^{-8}M	0.565	0.573	0.580
Ca4 μM	0.568	0.577	0.583
Ca8 μM	0.564	0.573	0.580
K6 μM	0.603	0.613	0.624
K12 μM	0.629	0.636	0.641
Ca4 + IAA	0.570	0.583	0.596
Ca8 + IAA	0.569	0.579	0.586
K6 + IAA	0.613	0.626	0.633
K12 + IAA	0.639	0.647	0.656
IAA + Ca4	0.579	0.589	0.597
IAA + Ca8	0.583	0.593	0.604
IAA + K6	0.636	0.651	0.663
IAA + K12	0.660	0.673	0.677
C.D. at 5%	0.021	0.018	0.023

Chapter-5

Discussion

CHAPTER-5

DISCUSSION

The process of seed germination starts with the imbibition of water and is accompanied with an increase in the metabolic activity. It is induced by the activation and the *de novo* synthesis of the hydrolytic enzymes (Bewley and Black, 1985). A larger share of these simpler substances, so produced, is carried to the embryonic axis to sustain its growth (Beevers, 1968). However, the rate and the direction of catabolic and anabolic reactions may, to some extent, be modified by selected chemicals including phytohormones (Davies, 1987) and the inorganic elements (Marschner, 1986).

A perusal of the observations (Table 1), like that of Tahir and Farooq (1989), revealed an increase in the activity of nitrate reductase as the germination process advanced. However, the contents of the nitrate (Table 2) showed a reverse trend, in confirmity with Egami *et al.* (1957). The NR is an inducible enzyme. Out of the factors which regulate the level of NR include the presence or absence of the substrate (Hewitt and Afridi, 1959; Afridi and Hewitt, 1964) and the phytohormones (Roth-Bejerane and Lips, 1970; Ahmad 1994, Ahmad and Hayat, 1999; Hayat *et al.*, 2001). In the present study, the seeds were simply soaked in distilled water/ a specific concentration of the auxin and/or the inorganic ions (Ca^{2+}/K^{+}). The germinating seeds therefore, totally depended, for its nitrate requirement, on the reserves. Under these uniform conditions, the IAA and/or the

cations (Ca^{2+}/K^{+}) elevated the level of the enzyme in the germinating seeds of *Cicer arietinum*. The auxin alone proved to be most effective in inducing the activity of the enzyme by possibly involving the genes because the synthesis of this enzyme involves the synthesis of both mRNA and protein (Jones and Prasad, 1992). The increase in the cytosolic level of calcium is known to trigger the cellular response (Stryer, 1992). Therefore, one of the observed expressions of the increase (Tables 4 & 5) in either of the ions (K^{+} or Ca^{2+}) is the elevated level of NR. The other enzyme (α-amylase) is known to have a direct association with the level of the Ca^{2+}, during germination of rice seed (Ying *et al.*, 1996). The impact of the K^{+} on the level of NR is possibly because of its involvement as an activator, co-factor in about 46 enzymes (Evans and Sorger, 1966). The treatment of the seeds with the either of the ions before or after being fed with auxin proved fruitful but the values of NR activity were less than those generated by the auxin alone. This variation may be due to the difference in the soaking pattern because in the former the duration of auxin treatment was just the half of the latter.

The nitrate content (Table 2) decreased linearly with the advancement of the germination as it was not supplemented from outside in the soaking medium. However, the rate of the loss (reduction) varied with the treatment but maximum quantity was found in the control at each stage of sampling. The nitrate content and the NRA showed inverse relationship with each other. It is obvious from these observations that a larger part of the nitrate was reduced under high NR activity. Therefore,

NR may be an appropriate marker enzyme for defining the importance of reduced nitrogen in the seed to takeup and reduced nitrate to nitrogen.

The major rate limiting stage in the reduction of nitrate to ammonical nitrogen for being incorporated in the production of amino acids is the very first step where nitrate is reduced to nitrite and is regulated by the enzyme NR (Hopkins, 1995). It is, therefore, understood that the increase in the activity of NR by the treatment (Table 1) will obviously enhance the availability of organic nitrogen and in turn that of amino acids. It possibly facilitated the synthesis of specific enzymes required for the synthesis of proteins (Hopkins, 1995) where its level had a concomitant increase (Table 3) with an increase in the level of NR. There seems to be a parallel relationship between NR and the level of the protein which is also supported by Singh and Singh (1985). The possible sites where the hormone and the ions could have acted is either at the level of the membrane and/or that of the genes.

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Appendix

APPENDIX

PREPARATION OF REAGENTS

The various reagents used for biochemical determinations were prepared according to the following methods.

1.0 Reagents for the estimation of nitrate reductase activity

1.1 Phosphate buffer (pH 7.5)

(a) 13.6 g potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in sufficient double distilled water and the final volume was adjusted upto 1 litre.

(b) 17.42 g dipotassium monohydrogen orthophosphate (K_2HPO_4) was dissolved in sufficient double distilled water and the final volume was made upto 1 litre.

(c) 160 ml of solution (a) and 840 ml of solution (b) was mixed in order to get pH 7.5.

1.2 Potassium nitrate (0.2M)

2.02 g potassium nitrate was dissolved in enough double distilled water and the final volume was made upto 100 ml.

1.3 Sulphanilamide solution (1%)

1 g sulphanilamide powder was dissolved in 100 ml 3N HCl.

1.4 NED-HCl solution (0.02%)

20 mg NED-HCl N-1-(naphthyl)-ethylene diamine dihydrochloric acid was dissolved in 100 ml double distilled water.

2.0 Reagent for the estimation of nitrate

2.1 Phenoldisulphonic acid reagent

25 g pure white phenol (AR) was dissolved in 150 ml of pure concentrated sulphuric acid to which 75 ml fuming sulphuric acid was added (13% SO_3). The solution was heated for about 2 hours at 100°C in water-bath and cooled. This solution was kept in a dark bottle in refrigerator.

3.0 Reagent for the estimation of protein

3.1 Reagent D

50 ml of 2% sodium carbonate was mixed with 1 ml of 0.5% copper sulphate and 1% sodium tartarate (which is prepared in the ratio of 1:1).

3.2 Folin's phenol reagent

100 g sodium tungstate and 25 g sodium molybdate was dissolved in 700 ml distilled water in which 50 ml of 85% phosphoric acid and 100 ml concentrated hydrochloric acid was mixed. The flask was connected with a reflux condenser and

boiled gently on a heating mantle for 10 hours. At the end of the boiling period, 150 g lithium sulphate, 50 ml double distilled water and 3-4 drops of liquid bromine was added to this flask. The reflux condenser was removed and the solution in the flask was boiled for 15 minutes in order to remove excess bormine, cooled and diluted to 1 litre.

The strength of this acid solution (1N) was tested by treating it with 1N sodium hydroxide using phenolphthalein as an indicator.